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REVIEW

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New frontiers in modeling tuberous sclerosis with human stem cell-derived neurons and brain organoids

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Abstract

Recent advances in human stem cell and genome engineering have enabled the generation of genetically defined human cellular models for brain disorders. These models can be established from a patient's own cells and can be genetically engineered to generate isogenic, controlled systems for mechanistic studies. Given the challenges of obtaining and working with primary human brain tissue, these models fill a critical gap in our understanding of normal and abnormal human brain development and provide an important complement to animal models. Recently, there has been major progress in modeling the neuropathophysiology of the canonical "mTORopathy" tuberous sclerosis complex (TSC) with such approaches. Studies using two- and three-dimensional cultures of human neurons and glia have provided new insights into how mutations in the *TSC1* and *TSC2* genes impact human neural development and function. Here we discuss recent progress in human stem cell-based modeling of TSC and highlight challenges and opportunities for further efforts in this area.

KEYWORDS

astrocytes, brain organoids, cortical tuber, CRISPR/Cas9, disease modeling, human pluripotent stem cells, mTOR, neurons, TSC1, TSC2, tuberous sclerosis complex

1 | INTRODUCTION

1.1 | Clinical presentation of TSC

Tuberous sclerosis complex (TSC) is a multisystem developmental disorder with a prevalence of 1 in about 6000 births worldwide. TSC causes benign tumors, called hamartomas, in multiple organs including the skin, lungs, kidney, and brain.[1] Hallmark pathologies of TSC are cortical tubers, which are focal developmental malformations consisting of enlarged and dysplastic neurons, glia, and giant cells in the cortex.[2] TSC is associated with significant neurological and psychiatric impairments, which include epilepsy in about 80% of individuals and variable degrees of intellectual disability.[3] TSC patients also have high rates of autism spectrum disorder, attention deficit hyperactivity disorder, and other behavioral and affective disorders.[4] Epilepsy is a major concern in TSC as it can begin in infancy and becomes intractable in about two-thirds of patients.[5] In some cases, surgical resection of the affected brain tissue is required to mitigate seizures.

TSC can be treated with rapamycin (also called sirolimus) and its derivative everolimus, collectively known as rapalogues. Recent clinical trials with these drugs have shown benefit for treating epilepsy in TSC, with approximately 40% seizure reduction in 40% of individuals.[6] Rapalogues are also effective at treating the glioneuronal brain tumors that occur in about 5% to 20% of TSC patients, called subependymal giant cell astrocytomas, or SEGAs.[7] However, tumors may regrow if treatment is

stopped.[8] Clinical trials with rapalogues focusing on neuropsychological deficits and autistic symptoms in TSC are underway (www.clinicaltrials.gov), although a recent trial did not report significant improvements in neurocognitive functioning with six months of daily everolimus.[9] Systemic side effects associated with chronic rapalogue use are prevalent but generally tolerated and include infections due to immunosuppression and stomatitis.[6] Despite some success with rapalogues, there is still an unmet clinical need for TSC treatment, and additional therapeutic approaches are required.

1.2 | Biochemistry and genetics of TSC

TSC is caused by loss-of-function mutations in the *TSC1* or *TSC2* genes.[10,11] These genes encode the proteins hamartin (TSC1) and tuberin (TSC2) that together with TBC1D7, form a multimeric protein complex,[12] which represses mTOR complex 1 (mTORC1) signaling. Biochemically, TSC2 is a GTPase-activating protein (GAP) for the small GTPase Rheb, which prevents mTORC1 activation.[13] TSC1 is a stabilizer of TSC2, preventing its degradation and enhancing its GAP activity.[13] As an active signaling node, mTORC1 promotes anabolic cellular processes through a multitude of functions, including stimulation of protein and lipid synthesis, cellular metabolic control, and suppression of autophagy, among others.[14,15] Constitutive or deregulated mTORC1 activity, as in the case of TSC1/2 complex loss, causes increased cell growth and altered proteostasis.[16] Loss of either TSC1 or TSC2 is sufficient to cause mTORC1 hyperactivity. However, loss-of-function mutations in *TSC2* tend to cause greater mTORC1 activation and are associated with more severe epilepsy and cognitive impairment.[17-21]

Patients with TSC have germ line heterozygous mutations in *TSC1* or *TSC2*; however, pathological lesions including cortical tubers are variable and appear stochastically. A leading hypothesis to explain cortical tuber formation is the "two-hit" model.[22] This model proposes that loss of heterozygosity due to a somatic second-hit mutation in *TSC1* or *TSC2* in a small population of neural progenitor cells (NPCs) causes altered differentiation, development, and neuronal migration, resulting in a focal malformation. In support of this model, second-hit mutations in *TSC1* or *TSC2* are frequently identified in TSC-related tumors and have been detected in some cortical tubers.[23-30] In addition, studies in mouse and human cellular models have shown that complete loss of TSC1/2 function is required for the formation of enlarged, dysplastic neurons and glia, which do not develop from a heterozygous state.[21,31,32] That said, this idea has been controversial in the field, as second-hit mutations have been identified in only a minority of tubers tested.[23,28-30] This may reflect low allelic frequency of the somatic mutation,[33] dilution of the second-hit cells by infiltrating glia, mutations in regulatory regions that are not assessed in exome sequencing studies,[34] or epigenetic changes.

1.3 | Other mTORopathies

TSC is representative of a larger class of disorders called "mTORopathies," caused by mutations in mTOR pathway regulators, that result in constitutive activation of mTORC1 signaling. In addition to TSC1 and TSC2, these regulators include PTEN, PI3K, AKT3, DEPDC5, STRADA, Rheb, and others.[35,36] Recently, it was shown that in addition to germ line mutations, mTORopathies can arise through somatic brain mutations, including gain-of-function mutations in mTOR itself.[33,37-42] These somatic mTORC1-activating mutations have been identified in brain tissue from patients with focal cortical dysplasia and hemimegalencephaly. Notably, an mTOR-activating mutation detected in less than 10% of brain cells is sufficient to cause disease.[33] These types of somatic mutations are more challenging to model than inherited mutations as they require a mechanism to induce them in a sparse population of cells and early in brain development. Given potentially similar underlying neuropathophysiology, disease mechanisms discovered in models of TSC are likely to provide insights into this greater class of mTOR-related disorders.

1.4 | Rodent models of TSC

Rodent models have provided key insights into the consequences of Tsc1/2 loss on brain development and function. Germ line knockout (KO) mouse models demonstrated that complete loss of *Tsc1* or *Tsc2* is embryonic lethal,[42,43] and subsequent conditional KO models of *Tsc1* and *Tsc2* have been developed.[44,45] Different applications of Cre recombinase using viral injections, in utero electroporation, or Cre-expressing mouse lines have illuminated the effects of Tsc1 or Tsc2 loss on multiple neuronal types. Somatic mTOR-activating mutations have also been modeled in mice by sparse expression of mutant *MTOR*, constitutively active Rheb, or CRISPR/Cas9 constructs targeting *Tsc1* or *Tsc2*.[46-49] The Eker rat model of TSC, carrying a spontaneous loss-of-function mutation in *Tsc2,* was shown to develop sporadic cytomegalic neurons, glia, and SEGAs in aged or irradiated young animals to induce a second hit.[50-52]

Collectively, these rodent studies have shown that loss of Tsc1/2 function impacts multiple processes happening at different developmental time points. These include altered neuronal differentiation, survival, migration, morphology, excitability, synaptic plasticity, glial function, and behavior (for relevant reviews, see Tsai and Sahin,[53] Costa-Mattioli and Monteggia,[54] Crino et al,[55] Magri and Galli,[56] and Lipton and Sahin[57]). In general, complete loss of *Tsc1* or *Tsc2* is required to observe strong disease-related phenotypes. That said, heterozygous

animals do exhibit some changes in synaptic function, neuronal excitability, and behavior.[58-61] While these rodent models have been and will continue to be powerful research tools, it is important to note that bona fide cortical tuber regions are not readily observed in animal models, suggesting that this pathology may result from unique aspects of human brain development.

1.5 | Opportunities for human stem cell-based models of TSC

While the rodent and human brain exhibit overall similar developmental patterns and trajectories, there are unique aspects of human brain development that cannot be captured in animal models. In particular, the human cortex develops over a significantly protracted time period compared to that of mice and exhibits unique cell types and proliferative zones.[62] For example, the dramatically increased complexity and size of the human cortex is thought to be due to a specific progenitor cell type called outer radial glia that forms the outer subventricular zone, which is not present in rodents.[63,64] Notably, outer radial glia have been shown to exhibit high levels of mTORC1 activity and unique expression of mTOR-pathway components,[65] indicating an important role for mTOR signaling in human cortical development. The human brain comprises about 85 billion neurons, and at the peak of neurogenesis 100,000 new neurons are generated each minute.[66] This massive cell proliferation also results in increased liability for somatic mutations, whose contribution to both normal and abnormal human brain development is becoming increasingly appreciated.[66]

To capture these unique aspects of human brain development and understand how alterations in TSC-mTOR signaling affect these features, human cellular models are needed. Recent advances in somatic cell reprogramming have allowed the derivation of human-induced pluripotent stem cells (hiPSCs) from skin or blood cells from patients, which have the advantage of preserving patient-specific genetic information.[67] With the advent of site-specific nucleases as gene-editing tools, most notably CRISPR/Cas9, human pluripotent stem cells (either hiPSCs or human embryonic stem cells [hESCs]) can be genetically engineered with good efficiency to generate disease models with targeted mutations.[68-70] Combining these approaches, it is also feasible to genetically engineer patient-derived cells to either correct the mutation or introduce an additional mutation, thereby establishing a genetically controlled, isogenic disease model using a patient's own cells.[71] These stem cells can subsequently be differentiated into numerous somatic cell types, including neurons and glia, for modeling brain disorders.[72]

Depending on the differentiation protocol employed, two- or three-dimensional cultures comprising different lineages of neurons and glia can be established. Cortical excitatory neurons and astrocytes of the telencephalic lineage are key cell types of relevance for TSC, as the dysplastic cells in tubers are positive for glutamatergic and astrocytic markers.[55] These cell types can be generated through manipulation of endogenous neuroectodermal differentiation pathways via either inhibition of the dual-SMAD pathway[73] or overexpression of transcription factors.[74] Studies with cell type-specific conditional KO mice have also highlighted cerebellar Purkinje cells as relevant to TSC pathophysiology, particularly the behavioral symptoms of autism.[61,75,76] A human cellular differentiation protocol based on the addition of specific growth factors has recently been established for cerebellar Purkinje cells and specifically applied to disease modeling in TSC,[77] as discussed in section 2.2 below.

While differentiation has traditionally been done in two-dimensional (2D) monolayer cultures, protocols have recently been adapted for three-dimensional (3D) differentiation to generate brain spheroids or organoids (collectively called brain organoids here).[78-81] As discussed by Chen et al in this special issue, 3D brain organoid models have advantages over 2D models, including more complex cytoarchitecture and cellular niches that preserve cell–cell and cell-matrix interactions.[82]

The approach of differentiating neurons and glia from human pluripotent stem cells generally operates on a human developmental timescale. For example, by transcriptional profiling, a two- to three-month-old human brain organoid is roughly equivalent to a 16- to 19-post-conception-week human brain.[80] This enables the observation and manipulation of human neural development in approximately real time. For this reason, neurodevelopmental disorders such as TSC are particularly well suited to this disease modeling approach. In the next section we will describe published work to date using human stem cell-derived neurons and brain organoids to investigate disease mechanisms in TSC.

2 | HUMAN NEURON AND BRAIN ORGANOID MODELS OF TSC

2.1 | Forebrain excitatory neurons and glia in 2D cultures

2.1.1 | Alterations in differentiation, signaling, and gene expression

Initial work in developing human neuronal models of TSC has focused on the differentiation of genetically engineered hESCs,[21,83,84] TSC patient-derived iPSCs,[85-87] or gene-edited TSC iPSCs[88] into 2D forebrain cultures. These studies were undertaken using a variety of neuronal differentiation methods, investigating the effects of *TSC1* or *TSC2* reduction on neural precursors, neurons, astrocytes, and, in one case, oligodendrocytes.[87]

Differentiation into neural precursors proceeded normally in each study with only minor differences observed, such as increased neural rosette size in $TSC2^{-/-}$ cultures[83] and

increased proliferation rate in *TSC2*$^{+/-}$ cultures,[85] although this was not observed in other studies.[86] In contrast to this normal early neural differentiation, terminal differentiation into neurons proved highly problematic for cells with complete loss of TSC1/2 complex function. Specifically, *TSC2*$^{-/-}$ cultures produced significantly lower numbers of cells expressing the neuronal markers HuC/D.[83] Notably, loss of one copy of *TSC1* or *TSC2* was much less deleterious with cultures exhibiting either a minor decrease in HuC/D-positive cells[83,86] or no decrease at all.[87] The differentiation defects in cells with loss of TSC1/2 may be due to a combination of increased neuronal death,[83] delayed neuronal differentiation,[86] or a shift toward astroglial fate.[83,84] Dissecting the potential mechanisms of altered differentiation will be an interesting avenue for future investigation with these models.

The expected hyperactivation of mTORC1, as indicated by increased phosphorylation of downstream targets, including ribosomal protein S6, was observed in all studies. However, the strong effects seen at every developmental stage in *TSC2*$^{-/-}$ cultures[83] were not consistently seen at the NPC stage in *TSC2*$^{+/-}$ cultures.[21,86] Transcriptome analysis through RNA sequencing of patient iPSC-derived heterozygous NPCs found 513 differentially expressed transcripts compared to a sibling control line. Gene ontology analysis indicated that these transcripts were primarily involved in neuron migration and development.[86] Independent RNA sequencing of isogenic, gene-edited *TSC2* heterozygous and homozygous cultures found very few differences between *TSC2*$^{+/-}$ and *TSC2*$^{+/+}$ cells (10 transcripts) but large differences between *TSC2*$^{-/-}$ and *TSC2*$^{+/+}$, with more than 2000 transcripts differentially expressed.[84] It is possible that some of the differences between the patient iPSC and control line in the study by Zucco et al could be driven by genetic differences independent of the *TSC2* mutation. Analysis of additional TSC patient and control cell lines would be helpful to resolve this. In the *TSC2*$^{-/-}$ cultures in the Grabole et al study, groups of transcripts involved in astrogliosis, inflammation, and glycolysis were all up-regulated, which corresponds to observations of poor mitochondrial function in gene-edited iPSC-derived *TSC2*$^{-/-}$ neurons.[88] The transcriptome of *TSC2*$^{-/-}$ neural cultures also closely corresponded with previous microarray studies of cortical tubers and SEGAs.[89,90]

Given the key involvement of mTORC1 signaling in mRNA translation, translational profiling may reveal further differences in TSC neural cultures that may occur independently of transcriptional changes. This will be an interesting avenue for future exploration. Related to this, a recent study showed that mTORC1 signaling and translation of the translational machinery are both high in human pluripotent stem cells but suppressed during neural differentiation.[91] In addition, numerous changes in mRNA translation without a corresponding change in mRNA levels were observed across human neuronal development, highlighting the importance of translational control for developing neurons.[91]

2.1.2 | Impact on neuronal morphology and physiology

The most dramatic morphological differences were observed in homozygous *TSC2* KO cells. *TSC2*$^{-/-}$ NPCs, neurons, and glia exhibited somatic hypertrophy, and neurons displayed increased dendritic arborization.[21,83] The effects of heterozygous *TSC1* or *TSC2* loss were less clear for these cultures, with either no change in neuronal morphology,[83,86] minor increases in dendritic branching and no change in soma size,[87] or increases in both.[85] One note is that the study by Li and colleagues was based on a single cell line from one TSC patient compared to an iPSC line from an unrelated individual. It therefore remains to be determined whether phenotypic differences between these cell lines are due to the *TSC2* mutation or a result of cell line variability or genetic background.

Electrophysiological phenotypes were probed in a subset of studies with either whole-cell electrophysiology, multielectrode arrays (MEA), or calcium imaging.[83,87] Whole-cell recordings showed a strong decrease in the frequency of spontaneous (sEPSCs) and miniature (mEPSCs) excitatory postsynaptic currents in both *TSC2*$^{+/-}$ and *TSC2*$^{-/-}$ neurons in a gene dose-dependent manner.[83] However, mEPSC amplitude was increased in *TSC2*$^{-/-}$ neurons, suggestive of increased synaptic strength. *TSC2*$^{-/-}$ but not *TSC2*$^{+/-}$ neurons also had significantly reduced intrinsic excitability, consistent with their morphological alterations and changes in passive membrane properties.[83] This decreased intrinsic and synaptic excitability in developing *TSC2*$^{-/-}$ neurons suggests that other circuit components, for example, inhibitory neurons, may be required to generate hyperexcitability at the network level following loss of TSC1/2 function.[92,93] By contrast, MEA recordings of heterozygous cultures from patient TSC iPSCs did show increased spontaneous network activity, which was also reflected by the increased frequency, but not amplitude, of calcium transients in these cultures.[87] Discrepancies between these findings may reflect gene dose-dependent effects, cell line and culture variability (which could have significant effects on network activity levels and development), or inhibitory and excitatory neuron composition of the cultures, which was not explored in these studies.

Treatment with rapalogues and other mTOR inhibitors such as AZD-8055 reversed many of the phenotypes of TSC1 or TSC2 loss in forebrain neural cultures, including altered electrophysiology,[83,87] aberrant morphology,[83,87] hyperactive mTORC1 signaling,[83,85,86] and altered mRNA translation.[84]

2.2 | Cerebellar Purkinje cells

While forebrain excitatory cultures deficient in *TSC1* or *TSC2* have been the primary focus of most studies thus far because of their potential to develop into cortical tuber-like cells, cerebellar tubers can also form in some TSC patients.[94,95] In addition, mouse studies have demonstrated the importance of Tsc1/2 function in cerebellar Purkinje cells for autism-related behaviors.[61,75,76] To generate a cerebellar model for TSC, a new human Purkinje cell differentiation protocol was developed, and hiPSC lines from three individuals with TSC were generated, using cells from the parents or unaffected individuals as controls.[77] In addition, this study made use of an established *TSC2* heterozygous patient iPSC line, which had been further genetically engineered to create a $TSC2^{-/-}$ cell line together with a repaired $TSC2^{+/+}$ control cell line.[88] This strategy has significant advantages over the use of control iPSC lines from unrelated individuals as it provides an isogenic system in which cells have the same genetic background and differ only in the disease gene.

In this model, many of the same phenotypes as in forebrain cultures were observed, including increased rates of NPC proliferation, up-regulated expression of astroglial markers, increased cell death, increased cell size, hyperactivation of mTORC1 activity, and decreased excitability of differentiated neurons.[77] These properties were observed in both heterozygous and homozygous cultures, with more severe deficiencies in $TSC2^{-/-}$ cells. Transcriptomic analysis again revealed more differential gene expression between homozygotes and controls than heterozygotes and controls, with similar differentially expressed transcripts as in forebrain cultures, including altered mitochondria and autophagy genes.[77,84] Interestingly, in cerebellar cultures there was also decreased expression of mRNA processing genes, including many genes that are targets of FMRP, the protein disrupted in the neurodevelopmental disorder Fragile X syndrome. Finally, treatment with mTOR inhibitors reversed all the observed phenotypic effects of complete *TSC2* loss.[77]

2.3 | 3D brain organoid models of TSC

Recent developments in 3D differentiation techniques to generate human stem cell-derived brain organoids provide a new platform to investigate neurodevelopmental disorders in a physiologically relevant setting that can be maintained for long periods of time.[78-81] Specifically, these models demonstrate some basic cortical patterning, including the presence of human-specific cellular niches[65] and neuronal migration.[96] These features may be particularly relevant for TSC, as cortical tubers are developmental malformations that reflect not only altered differentiation but also defective migration.

A recent study combined 3D neuronal differentiation with CRISPR/Cas9 genome editing to investigate the "two-hit" hypothesis of cortical tuber development in human brain organoids.[21] In addition to establishing a panel of hESC lines with constitutive loss-of-function mutations in *TSC1* or *TSC2*, the authors used CRISPR/Cas9 to create an hESC line with a constitutive mutation in one allele of *TSC2* and a conditional mutation in the other ($TSC2^{c/-}$). To generate a second hit, Cre recombinase was added to $TSC2^{c/-}$ brain organoids to cause biallelic inactivation of *TSC2* and expression of a red fluorescent protein at a defined point during development. Applying sub-saturating amounts of Cre recombinase at a stage when NPCs were proliferating resulted in populations of $TSC2^{-/-}$ cells that developed alongside $TSC2^{c/-}$ cells (which are effectively heterozygous), analogous to what is hypothesized to happen in the developing cortex of TSC patients.

Many of the developmental differences seen in 2D neuronal culture were also observed in this model, including a strong bias toward an astroglial cell fate, altered cell morphology, cytomegaly, and activation of mTORC1 signaling.[21] These phenotypes became more apparent over developmental time, with relatively minor alterations at the neural precursor stage and greater abnormalities following terminal neuronal or astrocyte differentiation. This may be because mTORC1 signaling is normally high in stem cells (hPSCs and NPCs) and becomes strongly suppressed during neurogenesis. Failure to suppress mTORC1 signaling during neuronal differentiation due to loss of the TSC1/2 complex may alter proteostasis and interfere with transcriptional and translational programs. Within the organoids, the second-hit mutation resulted in the cell-autonomous generation of multiple cell types found in cortical tubers, including dysmorphic neurons, dysplastic astrocytes, and giant cells. Comparisons between wild-type controls and cells heterozygous or homozygous KO for *TSC1* or *TSC2* revealed differentiation defects and mTORC1 hyperactivation only in organoids with homozygous deletion of *TSC1* or *TSC2*, supporting the two-hit model. In addition, phenotypes tended to be more severe in organoids with complete *TSC2* loss-of-function compared to *TSC1*.

Chronic treatment of brain organoids with rapamycin prevented mTORC1 hyperactivation and cytomegaly of $TSC2^{-/-}$ cells.[21] In addition, rapamycin treatment biased differentiation toward neuronal fates, indicating that mTORC1 signaling can bidirectionally control cell fate, and thus have a major impact on nervous system development. In addition, while early treatment with rapamycin shifted cell fate, later treatment did not, demonstrating a critical window for mTORC1 to regulate cell fate decisions in developing brain organoids. Removal of rapamycin after early treatment caused the return of mTORC1 hyperactivity in *TSC2* KO cells[21], indicating the potential necessity of chronic rapalogue use to fully treat TSC.

2.4 | Summary of human neural models of TSC

Taken together, the studies described above demonstrate the power of human stem cell-based disease modeling for neurodevelopmental disorders such as TSC. In particular, human cell studies have led to the robust generation of tuber-like cells, including giant cells, in vitro, enabling future studies into the molecular mechanisms for this developmental abnormality in the context of human-specific neural development. Collectively, these studies have revealed profound impairments in neuronal differentiation and development due to *TSC1/2* mutations together with increased production of astroglial cells. These findings are consistent with patient histology literature describing the altered cell morphology and molecular expression profile of cortical tuber cells.[54] In addition, findings in human neural models of TSC have supported the idea that alterations in astrocyte differentiation and function are important for TSC neuropathophysiology.[97] Thus, these initial studies have established and validated new models for TSC that can be employed to perform mechanistic investigations into how TSC-mTOR signaling contributes to early human brain development in both normal and pathological states.

In addition to allowing the study of human-specific cell biology, a key advantage of human cellular systems over rodent models is the ability to observe and manipulate the very earliest stages of neural development. This is technically challenging in mice, as germ line loss of *Tsc1* or *Tsc2* causes embryonic lethality prior to brain development. To circumvent this, brain-specific deletion of *Tsc1* or *Tsc2* can be utilized; however, this requires expression of Cre recombinase, which may not be expressed early enough in development to induce the full panoply of abnormalities that occur in utero in TSC. This could be a factor limiting the development of bona fide cortical tubers in rodent models. The ability to generate long-lived human neural cultures that develop from a stem cell state therefore provides a unique platform to perform early manipulations and to analyze their long-term consequences on neuron and astrocyte development.

3 | PERSPECTIVES, CHALLENGES, AND FUTURE DIRECTIONS

While human stem cell-based models for the neurological aspects of TSC have many compelling features, there are important caveats and considerations to this approach. In particular, while the ability to differentiate neurons from a stem cell state provides an opportunity to model the earliest stages of human brain development, it is important to note that these cells generally develop on a human timescale. Specifically, 3D brain organoids that are cultured for two to three months are transcriptionally similar to first- or second-trimester human fetal brain.[80] Therefore, the neurons and astrocytes in these models are reflective of an immature, fetal state. This is less of a problem, and indeed may be a useful feature, for studying abnormalities of brain development such as cortical tubers and related focal dysplasias. However, it can present a challenge when seeking to study aspects of TSC that may emerge later in development. Related to this, immaturity also applies to the functional properties of the neurons and astrocytes generated. For example, in 2D cortical cultures, neurons do not robustly fire action potentials until 7 to 10 weeks post-differentiation.[98] The maturation process can be accelerated by direct conversion of stem cells into functional neurons via forced expression of neurogenic transcription factors.[74] However, this type of protocol bypasses normal developmental stages that may be important to preserve in certain disease models. In brain organoids, mature electrophysiological activity is detected around 14 weeks and later,[99] and it may take more than six months post-differentiation for coordinated network activity to develop.[100] In terms of astrocyte function, a recent single-cell transcriptomics analysis of human cortical spheroids across development noted a shift in astrocyte gene signatures from fetal to postnatal around nine months in culture, with further maturation and development extending to spheroids grown for almost two years.[101] Therefore, if these more mature functional properties are required before pathophysiology emerges, human stem cell-derived neural cultures may need to be maintained for very long periods of time.

Another consideration is that while undirected brain organoid models that generate multiple cell types exist,[79] the variability and stochasticity with which different cell types are generated are a potential impediment to reproducibility required for disease modeling.[100] As a result, the majority of differentiation protocols are directed, in that they are designed to generate particular neural cell type(s) arising from a specific developmental lineage. One therefore needs to consider what cell types are most pertinent to the disease, and it may not be possible to have all relevant cell types present in a given model. This may be important in TSC, in which changes in several different cell types and non-cell autonomous effects may converge to produce disease phenotypes. For example, in mice, a growth factor secreted by Tsc1 KO neurons impairs oligodendrocyte development, which in turn negatively affects the myelination of cortical neurons.[102] In addition, in a *Tsc1* conditional KO mouse model, changes in inhibitory synapses onto excitatory neurons are what drive hyperactivity of the hippocampal network.[93] Neuronal differentiation approaches tend to produce mainly glutamatergic or GABAergic neurons but generally not both, as these arise from different lineages. Excitatory/inhibitory circuits have been modeled in 2D cultures by mixing separately derived excitatory and inhibitory neurons together,[103] or in 3D cultures by fusing pallium (excitatory glutamatergic lineage) and subpallium (inhibitory GABAergic lineage) organoids together into an "assembloid."[104]

A particularly exciting aspect of human stem cell-based disease modeling is the option to develop patient-specific models. These have the major advantage of preserving patient-specific genetic information. The disadvantage is the challenge in finding an appropriate control, as differences between cell lines generated from different individuals could reflect cell line variability or differences in genetic background unrelated to disease state. Gene editing approaches can be employed to generate isogenic cell lines with defined mutations. This approach can be done in the context of patient cells to capitalize on the advantages of both technologies, namely generating an isogenic disease model with patient-specific cells.[21,88] Advances in gene editing have also facilitated more sophisticated genetic models in which conditional mutations can be generated that were previously feasible only in animal models.[21,105] These conditional strategies may be particularly relevant to mTORopathies, as somatic mutations in the mTOR pathway can lead to disease.[33] In this case, using patient-derived cells to establish a model is not possible, as the somatic mutation would not necessarily be present in the fibroblasts or blood cells used for somatic cell reprogramming. Advanced gene editing can also be used for the expression of additional tools such as genetically encoded calcium indicators to monitor neuronal activity, optogenetics proteins for neuronal activation or silencing, or fluorescent cellular organelle reporters.[106]

In summary, the concurrent technical developments of neuronal differentiation from hPSCs, iPSC creation from human somatic cells, and genome engineering have facilitated the modeling of neurodevelopmental disorders in a laboratory setting. In particular, by applying these techniques to TSC, new human cellular models have been developed to not only answer questions about the developmental origins of the disorder but also give biological insights into treatment. We anticipate that the continued use and refinement of these models in conjunction with animal models will eventually lead to more effective and less invasive treatment for TSC and related mTORopathies.

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